Procedures for the Quantification of Human and Male Nuclear DNA

1 Scope

These procedures apply to DNA personnel who perform quantification to determine the quantity of amplifiable human and male nuclear DNA (nDNA) detected in a sample and DNA personnel that perform the associated quality control procedures. The DNA Casework Unit (DCU) and Scientific and Biometrics Analysis Unit (SBAU) use Sample Tracking and Control Software (STACS) and robotic workstations to automate the set-up of the quantification (aka quant) plates.

2 Equipment/Materials/Reagents

Equipment/Materials

- Tecan robot model 'Freedom EVO'
  - Tecan EvoWare software, version 2.3 or higher
- 7500 Real-Time PCR System, Applied Biosystems
  - HID Real-Time PCR Analysis Software vs 1.2 or higher
- STACS, version 3.2.920 or higher
- General laboratory supplies (e.g., pipettes, tubes)
- Microcentrifuge tubes (robot compatible)
- Speed-Vac, Vacufuge Concentrators, or equivalent
- 96-well Plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear plate seals
- Thermal Microplate Sealer

Reagents

- Quantifiler® TRIO DNA Quantification Kit
  - Quantifiler® TRIO DNA standard OR previously prepared dilution series
- Quantifiler® Automation Enhancer
- 007 sample, quantified and diluted as necessary
- TE Buffer (TE)
- 3% bleach (reagent grade or equivalent)
- 10% bleach (reagent grade or equivalent)
- Isopropyl alcohol, 70%
- Purified water or equivalent, available at laboratory sinks
- Water (reagent grade or equivalent)
- Roboscrub solution (Liquinox™ or equivalent)

3 Standards and Controls

The Quantifiler® TRIO DNA standard dilution series will be run in duplicate on each plate to generate the standard curve that is used to extrapolate the quantity of DNA in each sample.

A Master Mix control and TE control will be run on each plate. Evaluation of these standards and controls can be found in the Data Evaluation section of this procedure.

The 007 control is used as a positive control for troubleshooting purposes but there are no evaluation criteria for this sample.

The reagent blank(s) (RB) associated with each extraction batch are quanted to determine the RB with the greatest (if any) signal.

4 Procedures

Refer to the DNA Procedures Introduction (i.e., DNA QA 600) and follow applicable general precautions and cleaning instruction.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade, or equivalent, water will be used. The purified water, available via faucets (typically labeled DE) at the laboratory sinks, is used for Tecan operation and is also called Tecan system liquid.

4.1 Concentrating Extracted Samples Using the Speed-Vac

4.1.1	Samples may be concentrated using a Speed-Vac. • Samples from questioned items and corresponding RBs are generally reconstituted with reagent grade water, vortexed and quick spun prior to quantitation. Female fractions from vaginal swabs (and similar sample types) are generally not concentrated. • Known samples are generally not concentrated. The volume of water used to reconstitute, typically 15 µL or 25uL, will be recorded in the case notes. This volume is determined by the type of sample or as requested by the examiner. The volume used for the RB must be the same or less than the volume used for the associated samples.	

The Speed-Vac flask should be empty and dry, and the flask seal should be tight.

The Speed-Vac should be turned on ~45 minutes prior to use.

Ensure the gasket on the centrifuge is in its proper position and that the rotor is properly tightened prior to sample processing.

With the heat set to "High", a 100 µL extract may take ~1 hour dry. Samples should not be dried on "High" for more than four hours.

On the Speed-Vac with the heat set to "High", a 50 µL extract may take ~30-40 minutes to dry and a 100 µL extract may take ~60 minutes to dry. Samples should not be dried on "High" for more than four hours (maximum starting volume of ~400 µL).

On the Vacufuge with a setting of 60ºC, a 50 µL extract takes about 45 minutes.

4.2 Preparing the Tecan Robotic Workstation

If necessary, turn on the Tecan, which will undergo an initialization routine. Log on to the Tecan computer, launch and log on to the current Tecan software.

4.2.1	Ensure the Tecan is prepared to run: Prior to daily use: • Make ~100mL of 3% bleach to replace in front trough. • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script Prior to each run: • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. *When a carboy is refilled, it should be allowed to de-gas overnight before use.* • Check volume of waste container and empty if needed As needed: • Clean barcode scanners with a lint-free cloth	

The daily start up script prompt "Check syringes and tips" refers to checking that the tubing and syringes (plunger lock screws) are tight and not introducing air bubbles, and that the tips are tight, free of clogs, and not leaking.

4.3 Preparing the Sample Rack and Creating a Scan File Import

Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed from any network computer, as necessary.

Ensure all DNA extracts and reagent blanks (aka DNA sample tubes) are in Tecan compatible tubes and appropriately barcoded. Ensure all tubes are uncapped prior to run.

4.3.1	Place DNA sample tubes in positions 1 through 16 in the sample racks. Use additional sample racks as needed (up to 6 racks or 83 sample tubes). Any rack position(s) unfilled by a DNA sample tube must contain an empty tube with a unique "BL" barcode.	

"BL" barcode tubes may be reused; however, each "BL" barcode on the Tecan must be unique.

4.3.2	Use the current appropriate script to scan the sample racks and generate a .csv scan file. Import the file into STACS.	

4.4 Master Mix Preparation

This step may be performed any time prior to loading the master mix on the Tecan robot.

4.4.1	Enter appropriate barcodes into STACS. Create the master mix based on the volumes below. Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin.	

Quantifiler TRIO Master Mix Components

	µL per well*
PCR Reaction Mix	10.0
Primer Mix	8.0
Automation Enhancer	0.018**

*Number of wells = number of samples + 10 standards, 3 controls, and appropriate overage (~6)

NOTE: *Master mix must be created for a minimum of 56 wells to prevent pipetting less than 1 ul of automation enhancer.*

**Round the total volume of automation enhancer to 2 decimals as appropriate for the pipette capability.

4.5 Preparing the Tecan Deck

The below steps may be performed in any order prior to running the Tecan robot.

Positions of materials may vary between instruments. The robotic script will direct the placement.

4.5.1	Bleach/TE Rack: • Ensure the 3% bleach solution in the front trough was replaced prior to first daily use. • If making new standards, replace the ~100 mL TE in the center trough. Ensure that the trough has a "TE" barcode and is replenished as needed.	

4.5.2	If using the Tecan to reconstitute samples: Water Rack: • Ensure center trough has ~200 mL reagent grade water, replaced prior to each run.	

4.5.3	Place tubes in the Standards Rack (see Figure 1): • Positions 1 through 6: the Quantifiler® TRIO stock DNA standard solution* (with a unique "BL" barcode) and the standard dilution series tubes (with barcodes "S1" through "S5"). • Positions 7 through 14: empty tubes (with unique "BL" barcodes). • Position 15: a new uncapped tube (with a "TE" barcode). • Position 16: an uncapped 007 tube (with an "MC" barcode) containing at least 20 µL.	

Before loading, vortex, quick spin, and uncap the Quantifiler® TRIO stock DNA standard and/or the prepared standard dilution series, as appropriate.

Ensure the date the standard dilution series is prepared and biologist's initials are recorded on the standards (or standard rack). Prepared Quantifiler® TRIO standard dilution series may be used a maximum of five times or up to 5 days. The standards (or standard rack) will be labeled to indicate the number of times they have been used and refrigerated between uses.

*If using a previously prepared standard dilution series, any tube with a unique "BL" barcode (to include the stock standard tube) may be placed in position 1.

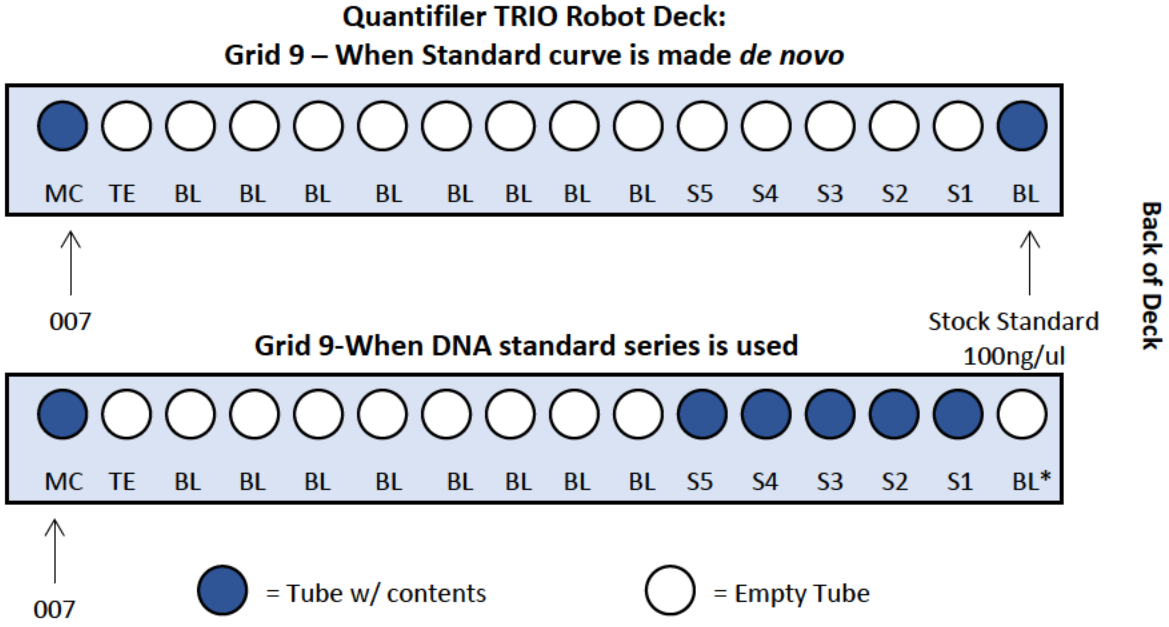

Figure 1 – Example Sample Positioning for Standards Rack

4.5.4	Plate Rack: • Place a 96-well plate into a base. Place into the front position of the plate rack with the A12 notch at back right. Ensure a quant batch barcode label is on the right side of the base or the plate, as appropriate.	

4.5.5	Place tubes in the Master Mix Rack (see Figure 2): • Positions 3 and 4: the two tubes containing equal volumes of master mix (with "C1" barcodes). Ensure tubes are uncapped. • Positions 1, 2, and 5 through 16: empty tubes (with unique "BL" barcodes).	

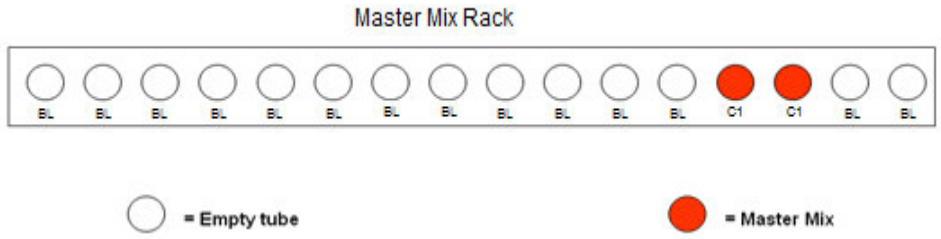

Figure 2 –Positioning for Master Mix Rack

4.6 Tecan Plate Preparation

4.6.1	Run the current version of the FBI Quantifiler TRIO script, and answer the prompts. The Tecan will add 18 µL of Quantifiler® TRIO master mix and 2 µL of each sample extract or control to the 96 well plate. The run takes ~20 minutes.	

4.6.2	Seal the plate with a clear seal. Quick spin (generally ~2,000 rpm for 5 seconds). Ensure the quant plate barcode is on a side of the plate.	

The seal may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are well sealed.

The DNA sample tubes and standard dilution series tubes should be removed from the Tecan deck and capped prior to taking the sealed quant plate to the Amp room.

4.7 Real-Time PCR

4.7.1	Ensure the 7500 and the supporting computer are powered on. Place the sealed plate into the 7500 so that well A1 is in the back-left and the notched corner of the plate is in the back-right.	

4.7.2	In the 7500 software, open a new run file. Import the sample setup (.txt file) generated by STACS for the plate ID.	

The .txt file can be found in the appropriate folder on the network.

4.7.3	Save the run file with the plate ID in the file name, ensure the 7500 door is closed, and start the run.	

Start the run from the Instrument tab or the Instrument menu.

The 7500 run generally takes ~1 hour.

4.8 Data Evaluation

4.8.1	Review the results in the 7500 software.	

The data may be analyzed from Amplification Plot of the Analysis Menu.

4.8.2	Review the Standard Curve plots of C_T (cycle threshold) versus Quantity (DNA concentration). [See Figures 3-5.] Use the Target dropdown menu to view the standard curve results for each target.	

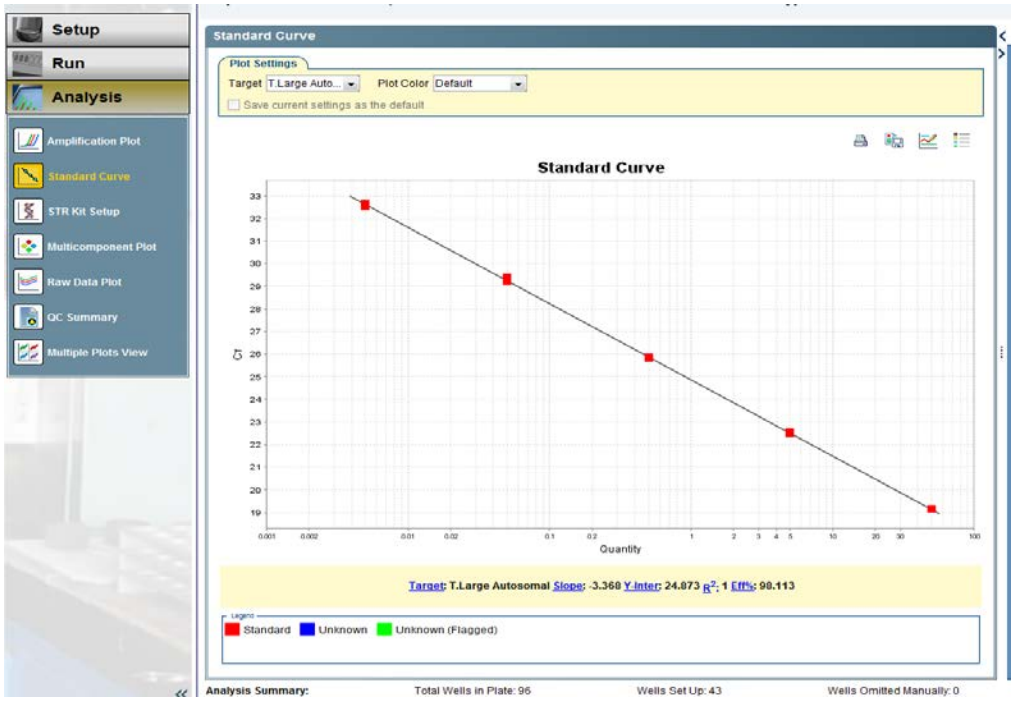

Figure 3 – Typical Standard Curve Demonstrating T. Large Autosomal TRIO Results

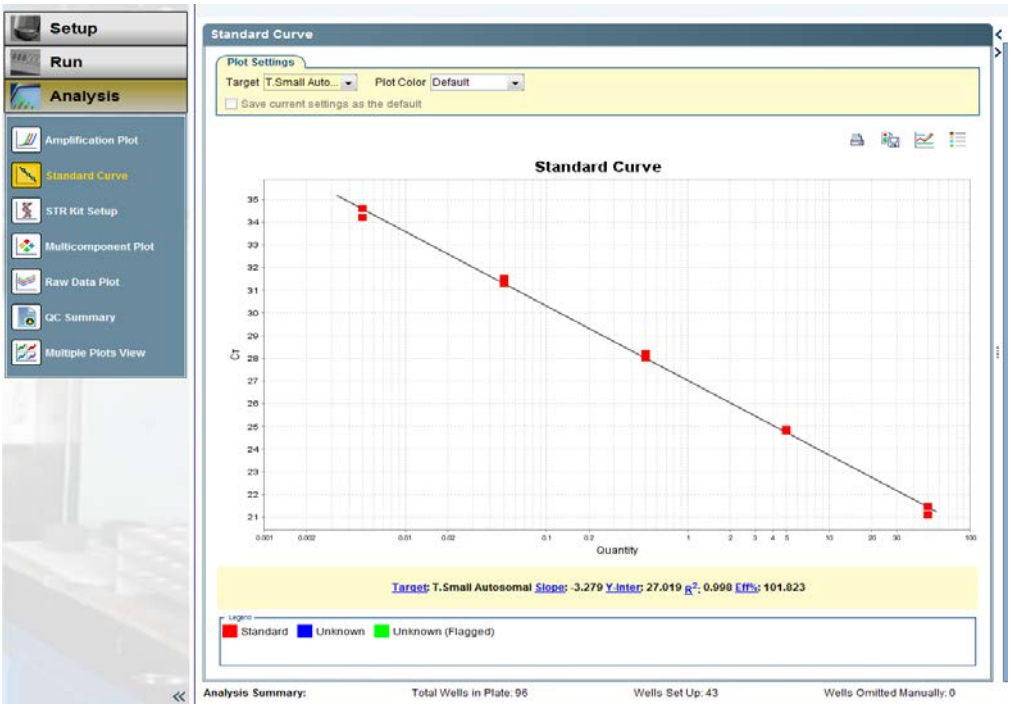

Figure 4 – Typical Standard Curve Demonstrating T. Small Autosomal TRIO Results

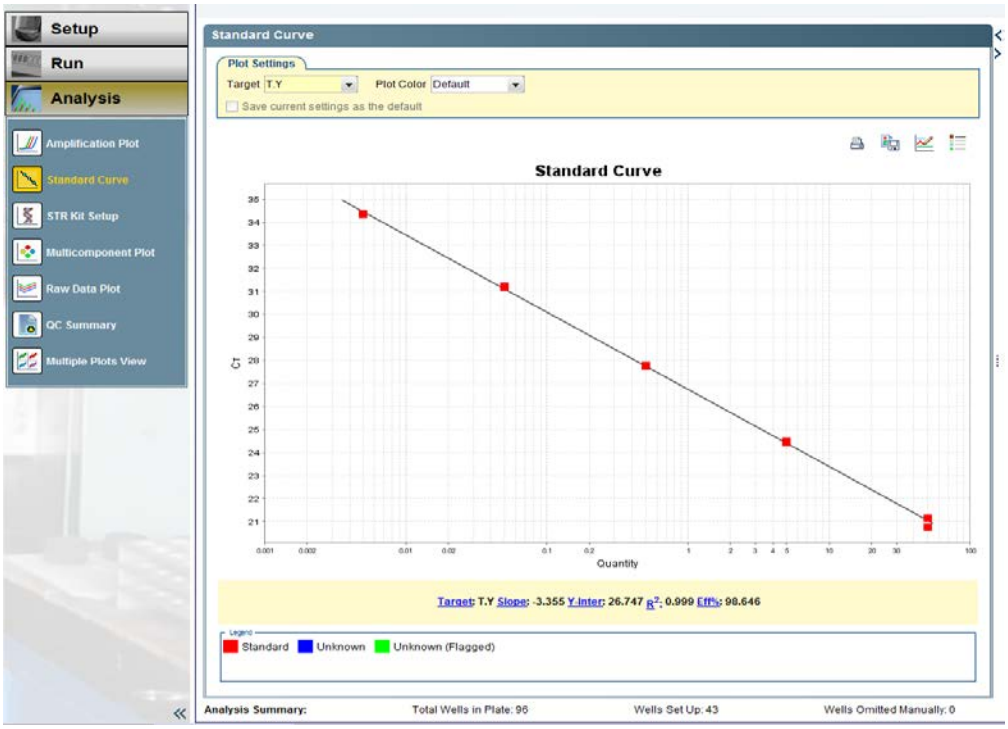

Figure 5 – Typical Standard Curve Demonstrating T.Y TRIO Results

4.8.2.1	A passing run will meet the standard curve parameters below:	

	Slope Range		**Y-intercept Range**		**R^2 Minimum**
T. Large autosomal	-3.664	-3.185	23.496	25.892	0.997
T. Small autosomal	-3.435	-3.115	25.678	27.716	0.996
T. Y	-3.574	-3.116	24.697	27.296	0.995

4.8.2.2	If the Y-intercept, R^2, or slope are out of range, or if there is a visible outlier, omitting a poor replicate of a standard and reanalyzing may improve the standard curve. (For each standard pair in the dilution series, only one of the replicates may be omitted if necessary).	

4.8.2.3	If the Y-intercept, R^2, and/or slope do not meet the required values after replicate omission, the plate fails, the data is not suitable for evaluation, and the samples must be re-quantified.	

If one or more replicates are deleted from the standard curve, or the Y-intercept, R^2, or slope fall outside the expected range, the standard dilution series used should be discarded.

4.8.3	Export the results to the appropriate file on the network. (*Select Export from File menu, then choose Results.*) Import the results file (.txt) into STACS. Enter the plate ID and the requested values for the T. Large Autosomal, T. Small Autosomal and T.Y assays (i.e., Y-intercepts, slopes, and R^2s) into STACS. This data transfer should be checked to ensure accurate transcription and who performed the check recorded in the notes. Import the .eds file into STACS.	

The tabulated data can now be reviewed in STACS.

4.8.4	Check the T. Large Autosomal, T. Small Autosomal and T.Y quantification results and IPC C_T of the Master Mix control. The Master Mix control should display no quantifiable DNA. If a DNA concentration value appears in the master mix control, the concentration values obtained for the RB(s) run on the plate should be examined. • If one or more of the RBs display no quantifiable DNA, the master mix value can be concluded to be spurious (i.e., not indicative of the presence of adventitious DNA) and the sample data should be used. • The T. Large Autosomal target is not used for quantification. Values appearing in this target alone should not be considered.	

4.8.5	For plates using a new DNA Standard dilution series, check the T. Small Autosomal and T.Y quant result and IPC C_T of the TE Control. The TE control should display no quantifiable DNA. • If a DNA concentration of ~0.010 ng/µL or less is detected, the sample data should be used. • If a DNA concentration between ~0.010 and 1 ng/µL is detected, the samples may be re-quanted. • If a DNA concentration of ~1 ng/µL or greater is detected, the samples must be re-quanted.	

4.8.6	The sample data can be evaluated to determine if any sample should be diluted and/or re-quanted. IPC C_T values are typically between 27 and 30. Undetermined IPC C_T values or values greater than 31 may indicate inhibition. • Samples that have an indication of possible inhibition may be diluted and re-quanted. • Samples with excessive DNA (generally >300 ng/µL) should be diluted and re-quanted.	

Reagent grade water is used to dilute samples as appropriate. Any dilution(s) made will be recorded in the case notes.

An examiner will review the quant results for each sample. STACS uses the quant results and the default amplification settings to determine the volume of sample to queue for amplification. An examiner should make adjustments to the amplification setup as necessary. Additional guidance is located in the nDNA amplification procedure in the *DNA Procedures Manual*.

5 Calculations

Not applicable.

6 Sampling

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

Based on internal validation studies, samples that fail to yield signal at quant will not yield amplification results. In rare instances, Trio may be affected by inhibition when amp kits are not. In such cases, it is possible that samples yielding no result at quant may yield DNA typing results.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the "Safe Work Practices and Procedures," "Bloodborne Pathogen (BBP) Exposure Control Plan (ECP)," "Personal Protective Equipment Policy," and "Chemical Hygiene Plan" sections of the *FBI Laboratory Safety Manual*.

9.2 Avoid reaching into the Tecan robot while it is running as personal injury could result from moving robot accessories.

10 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Safety Manual

DNA Procedures Manual

Applied Biosystems. *Quantifiler™ HP and Trio DNA Quantification Kits User Guide,* 2017.

Applied Biosystems. *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative CT Experiments*. 2010.

Applied Biosystems. *Installation and Maintenance Guide for the Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System*. 2006.

ARTEL. *MVS Multichannel Verification System User Guide.* 2006

Alfonina I, Zivarts M, Kutyavin I, et al. 1997. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res.* 25:2657-2660.

Green RL, Ines CR, Boland C, and Hennessy LK. 2005. Developmental validation of the Quantifiler real-time PCR kits for the quantification of human nuclear DNA samples. *J. Forens. Sci.* 50:809-825.

Higuchi R, Dollinger G, Walsh PS, and Griffith R. 1992. Simultaneous amplification and detection of specific DNA sequences. *BioTechnology* 10:413-417.

Higuchi R, Fockler C, Dollinger G, and Watson R. Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. *BioTechnology* 11:1026-1030.

Livak KJ, Flood SJ, Marmaro J, Giusti W, and Deetz K. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl.* 4:357-362.

Rev. #	Issue Date	History
4	05/25/16	Complete revision for simplification of procedure. Changed from nDNAU to DCU throughout. Changed from nDNAU LIMS to STACS thoughout and made necessary adjustments for STACS. Added 4.1 from extraction SOP since performed during quant. Moved QC procedures to Appendix and simplified.
5	02/28/18	1 Adjusted scope 2 Updated software and made necessary adjustments throughout 4.1.1 and 4.1.2 Allowed for both volumes typically used. Added clarification that volume is based on sample or as requested by FE. 4.5.4 Corrected numbering and added allowance for different placement of the barcode based on the Tecan plate holder. 4.8.2.1 Applies to human and male standard curves Appendix A: Edits so that standards in performance verification can be run in duplicate.
6	03/16/20	Complete revision to incorporate changes from Quantifiler DUO to Quantifiler TRIO. Moved RoboScrub to appendix A

Approval

Redacted - Signatures on File

DNA Technical Leader Date: 03/13/2020

DCU Chief Date: 03/13/2020

SBAU Chief Date: 03/13/2020

Appendix A: Quality Control Procedures

1. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency and additional requirements.

A. General Maintenance of the AB 7500 Real-Time PCR System

Once a year, general maintenance is performed as part of the annual PM. For semi-annual general maintenance, refer to the instructions in the Applied Biosystems *7500/7500 Fast Real-Time PCR System Maintenance Guide* to perform the following:

1. Regions of Interest (ROI) Calibration (Chapter 2)
2. Background Calibration and Optical Calibration (Chapter 3)
3. Dye Calibrations (Chapter 4) for standard dyes VIC and FAM and custom dyes ABY and JUN and, when applicable, for standard dye NED used for the mtDNA qPCR Degradation Assay.

B. Performance Verification of the AB 7500 Real-Time PCR System

The performance verification of the AB 7500 Real-Time PCR System will be accomplished by running both the Quantifiler® TRIO DNA Quantification Kit and, when applicable, the mtDNA qPCR Degradation Assay, as each assay uses different dyes.

1. Refer to the above Quantifiler® TRIO procedures and the procedures for the mtDNA qPCR Degradation Assay (i.e., DNA 404):
    a. Using an in-use lot of Quantifiler® TRIO kit, run a plate containing the standard dilution series and appropriate controls, all in duplicate.
    b. Using in-use lots of reagents for the mtDNA qPCR Degradation Assay, run a plate containing the mtDNA Quantitative PCR Standard Dilution Series, the HL60 calibrator, and appropriate controls, all in duplicate.

2. The 7500 will be deemed suitable for casework analysis if
    a. The slope, Y-intercept, and R^2 values for the Quantifiler® TRIO meet the criteria of a passing run:

	Slope Range		Y-intercept Range		R^2 Minimum
T. Large autosomal	-3.664	-3.185	23.496	25.892	0.997
T. Small autosomal	-3.435	-3.115	25.678	27.716	0.996
T. Y	-3.574	-3.116	24.697	27.296	0.995

    b. And the slope, Y-intercept, and R^2 values for the mtDNA qPCR Degradation Assay meet the criteria of a passing run:
        i. $R^2 \geq 0.985$
        ii. Slope in the range of -3.200 and -3.600
        iii. Y-intercept in the range of 36.100 and 39.600

3. If the performance verification of the 7500 does not meet the passing criteria for either assay, the unsuccessful plate(s) will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

C. General Maintenance of the Tecan Robotic Workstation

RoboScrub cleaning should be performed weekly, generally at the end of a workday:

1. Make ~3.5 L of diluted Liquinox (see instructions on the label of the bottle for preparation)
2. ~3.5 L purified water in a separate container is needed
3. Run the RoboScrub Clean script, and follow the prompts

D. Performance Verification of the Tecan Robotic Workstation

1. An Artel MVS Multichannel Verification System and NIST traceable standards will be used to test the accuracy and precision of the liquid handling by the Tecan. Refer to the *Artel MVS Multichannel Verification System User Guide* for operation of the Artel MVS.

2. The Tecan Robotic workstations are typically configured with eight (8) fixed tips and there are multiple volumes aliquoted during each procedure. A minimum of 6 repetitions will be performed with each tip for each volume.

3. The results must be within the tolerance limits set by DCU for each volume. At times, it may be necessary to modify/optimize the Tecan liquid class parameters (e.g., offset and factor).

4. If the performance verification of the Tecan does not meet the above listed criteria, the performance verification will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

2. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

A. Qualification of Applied Biosystems Quantifiler® TRIO DNA Quantification Kit

Each new lot of Quantifiler® TRIO kits will be evaluated by running a standard dilution series from the new lot in duplicate with appropriate controls. The new Quantifiler® TRIO kit lot will be deemed suitable for casework analysis if the slope, Y-intercept, and R^2 values meet the passing criteria in section 4.8.2.1.